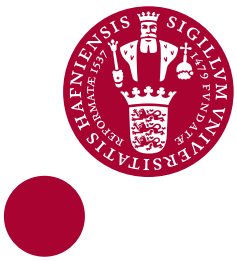

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Published in:
Antimicrobial Agents and Chemotherapy

DOI:
10.1128/AAC.02696-16

Publication date:
2017

Document version
Publisher's PDF, also known as Version of record

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Importance of the Exopolysaccharide Matrix in Antimicrobial Tolerance of *Pseudomonas aeruginosa* Aggregates

Lise Goltermann, Tim Tolker-Nielsen

Costerton Biofilm Center, Department of Immunology and Microbiology, University of Copenhagen, Copenhagen N, Denmark

ABSTRACT *Pseudomonas aeruginosa* is an opportunistic pathogen that can infect the lungs of cystic fibrosis (CF) patients and persist in the form of antibiotic-tolerant aggregates in the mucus. It has recently been suggested that such aggregates are formed due to restricted bacterial motility independent of the production of extracellular matrix components, and that they do not rely on an extracellular matrix for antimicrobial tolerance. However, we show here that biofilm matrix overexpression, as displayed by various clinical isolates, significantly protects *P. aeruginosa* aggregates against antimicrobial treatment. Alginate-overproducing *mucA* mutant bacteria growing in aggregates showed highly increased antibiotic tolerance compared to wild-type bacteria in aggregates. Deletion of *algD* in the *mucA* mutant strain abrogated alginate production and reversed the antibiotic tolerance displayed by the aggregates to a level similar to that observed for aggregates formed by the wild type. The *P. aeruginosa* Δ*wspF* and Δ*yfiR* mutant strains both overproduce Pel and Psl exopolysaccharide, and when these bacteria grew in aggregates, they showed highly increased antibiotic tolerance compared to wild-type bacteria growing in aggregates. However, the Δ*wspF* and Δ*yfiR* mutant strains, deficient in Pel/Psl production due to additional Δ*pelA* Δ*pslBCD* deletions, formed aggregates that displayed antibiotic tolerance levels close to those of wild-type aggregates. These results suggest that biofilm matrix components, such as alginate, Pel, and Psl, do play a role in the tolerance toward antimicrobials when bacteria grow as aggregates.

KEYWORDS biofilm, aggregates, extracellular matrix, antimicrobial tolerance

Received 21 December 2016 **Returned for modification** 15 January 2017 **Accepted** 22 January 2017

Accepted manuscript posted online 30 January 2017

Citation Goltermann L, Tolker-Nielsen T. 2017. Importance of the exopolysaccharide matrix in antimicrobial tolerance of *Pseudomonas aeruginosa* aggregates. Antimicrob Agents Chemother 61:e02696-16. https://doi.org/10.1128/AAC.02696-16.

Address correspondence to Tim Tolker-Nielsen, ttn@sund.ku.dk.

Microbial biofilms are typically viewed as aggregated bacteria embedded in an extracellular matrix composed mainly of polysaccharides, extracellular DNA, and proteins (1, 2). Work done mainly with surface-associated biofilms has suggested that bacteria in biofilms display increased antibiotic tolerance compared to planktonic microbes (3, 4). The studies suggest that a number of different mechanisms may be involved in antibiotic tolerance of biofilms, e.g., (i) restricted antibiotic penetration in cases where the antibiotic binds to components of the biofilm matrix, (ii) the presence of bacteria in various physiological states as a result of nutrient gradients in the biofilm, and (iii) expression of specific genes which promote antimicrobial tolerance of the bacteria in the biofilm (5, 6).

Pseudomonas aeruginosa is an opportunistic pathogen which has been extensively studied for its ability to form structured biofilm communities. *P. aeruginosa* infections, where the bacteria grow in the biofilm mode, occur typically in the lungs of cystic fibrosis (CF) patients and in chronic wounds, as well as on mechanical ventilator tubing and urinary catheters. When *P. aeruginosa* infects the lungs of CF patients, it persists in the form of aggregates in the mucus, and these are highly resilient to host immune responses and antimicrobial treatment (7).

P. aeruginosa strains isolated from chronic infections in CF patients show a distinct pattern of adaptation through mutation (8). Upregulation of the production of biofilm matrix components is a typical feature among clinical isolates, with phenotypes typically belonging to one of two major groups, the mucoid strains, which overexpress alginate, and the rugose small-colony variants (RSCVs), which mainly overexpress Pel and Psl polysaccharides.

Mucoid *P. aeruginosa* strains typically harbor inactivating mutations in the *mucA* gene, which in turn leads to activation of *algD* and production of alginate (9). Biofilms of mucoid *P. aeruginosa* strains are more tolerant to antibiotics than wild-type biofilms (10), and mucoidy has been implicated in increased tolerance to host immune responses (11).

The RSCVs massively overexpress the matrix components Pel and Psl, while pilus and flagellum genes are downregulated (12), resulting in a wrinkly colony morphology. Clinical RSCV isolates have been shown to be more tolerant to antimicrobials than their revertant counterparts (8, 13). Among the RSCV variants found in chronic lung infections are strains harboring loss-of-function mutations in *wspF* and *yfiR*, resulting in derepression of the di-guanylate cyclases WspR and YfiN, respectively. This in turn leads to increased production of the regulator molecule c-di-GMP and thereby increased production of biofilm matrix components, as well as a hyperadherent and autoaggregative phenotype (14, 15). The occurrence of RSCVs has been correlated with increased risk of failure of antipseudomonal treatment (16).

It has recently been demonstrated that *P. aeruginosa* bacteria can form aggregates independent of the production of exopolysaccharides in viscous environments that restrain bacterial motility, and it has been questioned if the biofilm matrix is at all important for protection against antimicrobial compounds when *P. aeruginosa* is present as aggregates in the viscous mucus of CF lungs (17).

In the present report, we provide evidence that biofilm matrix overexpression, as typically found in clinical isolates, provides protection of *P. aeruginosa* against antimicrobials when the bacteria are growing as aggregates. This might explain the strong selection for biofilm matrix-overproducing bacteria in chronic infections that are intensively treated with antibiotics.

RESULTS

Staudinger et al. (17) recently assessed the antimicrobial tolerance of *P. aeruginosa* aggregates formed in gels and found that the wild-type PAO1 and a Δ*pelA* Δ*pslBCD* Δ*algD* mutant strain displayed the same level of tolerance (17). Based on these results, it was suggested that the extracellular matrix does not play a role in the antimicrobial tolerance displayed by *P. aeruginosa* aggregates (17). Here, we performed investigations similar to those reported by Staudinger et al., but instead of comparing aggregate-associated antimicrobial tolerance of the *P. aeruginosa* wild type to that of mutants that do not produce extracellular matrix components, we compared the wild type to mutants that overproduce matrix components, as such mutants are frequently isolated from chronic infections.

Initially, we confirmed the observations reported by Staudinger et al. (17) by comparing PAO1 to a Δ*pelA* Δ*pslBCD* Δ*algD* knockout mutant strain. In accordance with the results of Staudinger et al., both strains formed visually detectable aggregates (300 to 500 μm) in the agar gel (Fig. 1A), suggesting that spatial confinement was sufficient for aggregate formation. Moreover, the aggregates formed by the wild type and the Δ*pelA* Δ*pslBCD* Δ*algD* mutant strain displayed the same level of tolerance toward tobramycin and ciprofloxacin (Fig. 1B).

Aggregates formed by mucoid *P. aeruginosa* display increased tolerance to antibiotics through alginate overproduction. We then investigated antimicrobial tolerance of aggregates formed by the mucoid *P. aeruginosa mucA* strain, which overproduces alginate because of an inactivating mutation in the *mucA* gene that normally suppresses alginate expression. The *mucA* strain formed aggregates in a manner similar to that of the wild-type PAO1 (Fig. 2A). Recovery of bacteria from

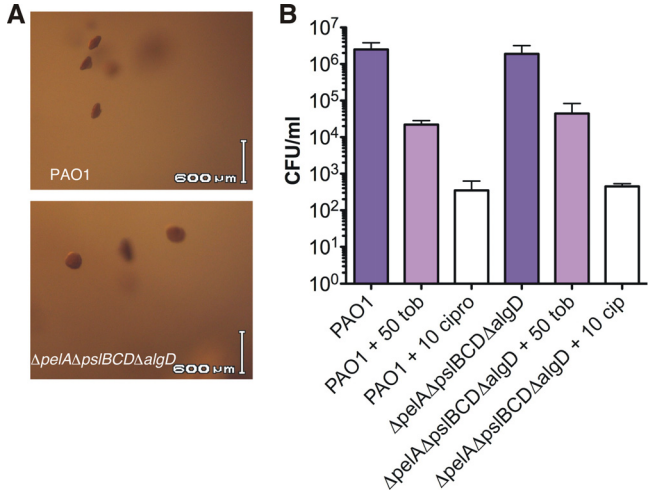

FIG 1 Aggregate formation and survival of *Pseudomonas aeruginosa* bacterial aggregates with matrix component deletions (Δ*pel* Δ*psl* Δ*alg*). Single bacterial cells were encapsulated in 0.8% LB agar and allowed to grow for 24 h to form bacterial aggregates. (A) Micrographs of gel plugs of PAO1 and of Δ*pelA* Δ*pslBCD* Δ*algD* mutant after overnight (o.n.) incubation in 0.8% LB agar. Each aggregate is approximately 300 to 500 μm. (B) Survival of PAO1 compared to Δ*pelA* Δ*pslBCD* Δ*algD* mutant. No statistical differences in survival were observed between the two strains upon antimicrobial treatment. The gel plugs were incubated for 3 h in saline without antibiotic (purple bars) or with either 50 μg/ml tobramycin (tob; magenta bars) or 10 μg/ml ciprofloxacin (cip or cipro; white bars), and bacteria were released from the gel and enumerated by plating; $n = 3$ to 5.

untreated *mucA* gel-encased aggregates was similar to recovery of bacteria from wild-type PAO1 aggregates, suggesting that the *mucA* aggregates, despite alginate overexpression, could be disassembled and the bacteria enumerated. Alginate overexpression provided a 50-fold increase in survival after treatment with tobramycin, while a 6-fold protection was measured for ciprofloxacin (Fig. 2B). This corresponds to observations done in surface-attached biofilms, where biofilms formed by mucoid *P. aeruginosa* have been shown to be more difficult to eradicate than the corresponding wild-type biofilms (18), while the MIC values for planktonic cultures were comparable (10). Deletion of the *algD* gene in the *mucA* background resulted in nonmucoid

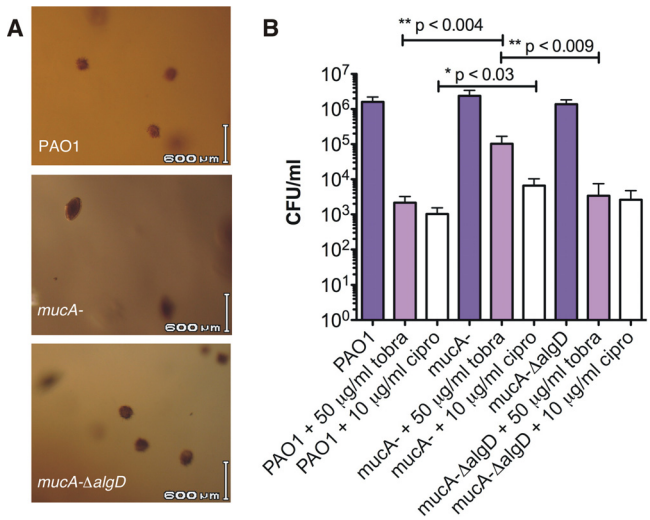

FIG 2 Aggregate morphology and bacterial survival of alginate-overproducing *Pseudomonas aeruginosa*. (A) Micrographs of aggregates encased in LB agar for PAO1, *mucA* mutant, resulting in mucoid colonies, and *mucA* Δ*algD* mutant nonmucoid colonies. (B) Survival of PAO1, *mucA* mutant, and *mucA* Δ*algD* mutant aggregates upon antibiotic treatment with 50 μg/ml tobramycin (tobra) or 10 μg/ml ciprofloxacin (cipro) for 3 h, $n > 4$. For details, see Fig. 1 legend.

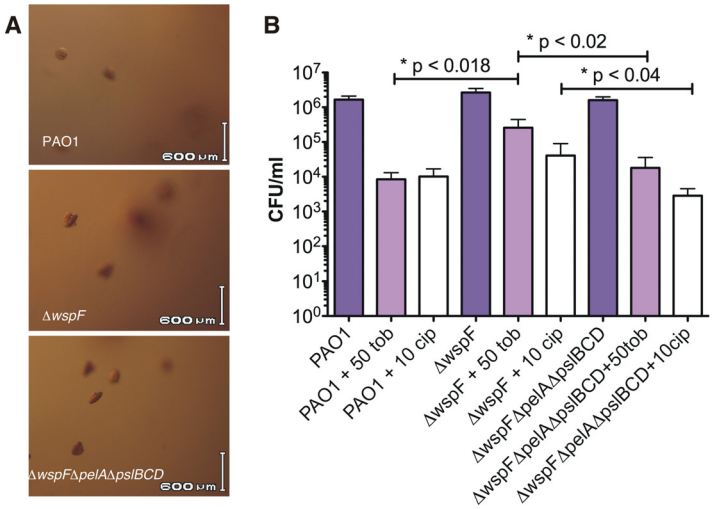

FIG 3 Aggregate morphology and bacterial survival of Pel/Psl-overproducing *Pseudomonas aeruginosa*. (A) Micrographs of aggregates of PAO1, Δ*wspF* mutant, and Δ*wspF* Δ*pelA* Δ*pslBCD* mutant. (B) Survival of PAO1, Δ*wspF* mutant, and Δ*wspF* Δ*pelA* Δ*pslBCD* mutant aggregates treated with 50 μg/ml tobramycin (tob) or 10 μg/ml ciprofloxacin (cip) for 3 h. For details, see the Fig. 1 legend.

bacteria, and aggregates formed by the *mucA* Δ*algD* mutant strain displayed the same level of sensitivity toward tobramycin as wild-type aggregates (Fig. 2B). Ciprofloxacin tolerance was also reversed, however, but not completely to the level of the wild-type aggregates.

Aggregates formed by *P. aeruginosa* RSCVs display increased tolerance to antibiotics through *pel* and *psl* overexpression. To assess the antimicrobial tolerance of *P. aeruginosa* aggregates which significantly overproduce Pel and Psl, we tested a Δ*wspF* mutant strain and a Δ*yfiR* mutant strain, which both overproduce c-di-GMP and biofilm matrix components and display an RSCV phenotype on agar plates and hyper-aggregation in planktonic cultures. In aggregates, this wrinkly colony morphology was also visible (Fig. 3A). We observed that bacteria in aggregates formed by the Δ*wspF* mutant strain displayed a 30-fold increase in survival after tobramycin treatment and a 4-fold increase in survival after ciprofloxacin treatment compared to bacteria in aggregates formed by the corresponding PAO1 wild type (Fig. 3B). We subsequently investigated if deletion of the *pel* and *psl* genes in a Δ*wspF* background could reduce the antimicrobial tolerance of the aggregates. The Δ*pelA* Δ*pslBCD* Δ*wspF* mutant strain growing in aggregates was significantly more sensitive to the tested antimicrobials than the Δ*wspF* mutant strain in aggregates (Fig. 3B), underlining the importance of the Pel and Psl matrix components in protection against antimicrobials in these aggregates.

We subsequently tested the RSCV strain harboring the Δ*yfiR* knockout mutation. The Δ*yfiR* mutant strain was the only of the tested strains which exhibited a lower recovery rate from the gel-encased aggregates compared to the wild type. We believe this might be caused by tighter aggregate formation. The Δ*yfiR* mutant aggregates had sizes similar to those of the wild-type aggregates but were visually distinguishable by their wrinkly surface (Fig. 4A). Therefore, survival data are presented both in absolute numbers as CFU per milliliter (Fig. 4B) and as fold reduction of antibiotic-treated compared to untreated aggregates (Fig. 4C). While tobramycin reduced the survival of the aggregate-forming wild-type bacteria by more than 150-fold, survival of the aggregate-forming Δ*yfiR* mutant bacteria was reduced only 5-fold. In the same way, ciprofloxacin reduced survival of the aggregate-forming wild-type bacteria by 2,500-fold and only 550-fold for the aggregate-forming Δ*yfiR* mutant bacteria. When we introduced the Δ*yfiR* deletion into a Δ*pelA* Δ*pslBCD* mutant background, we did not observe any difference in antibiotic tolerance between aggregates formed by PAO1 and the Δ*pelA* Δ*pslBCD* Δ*yfiR* mutant strain (Fig. 4D). This corresponds to what we observed for the Δ*wspF* mutant strain.

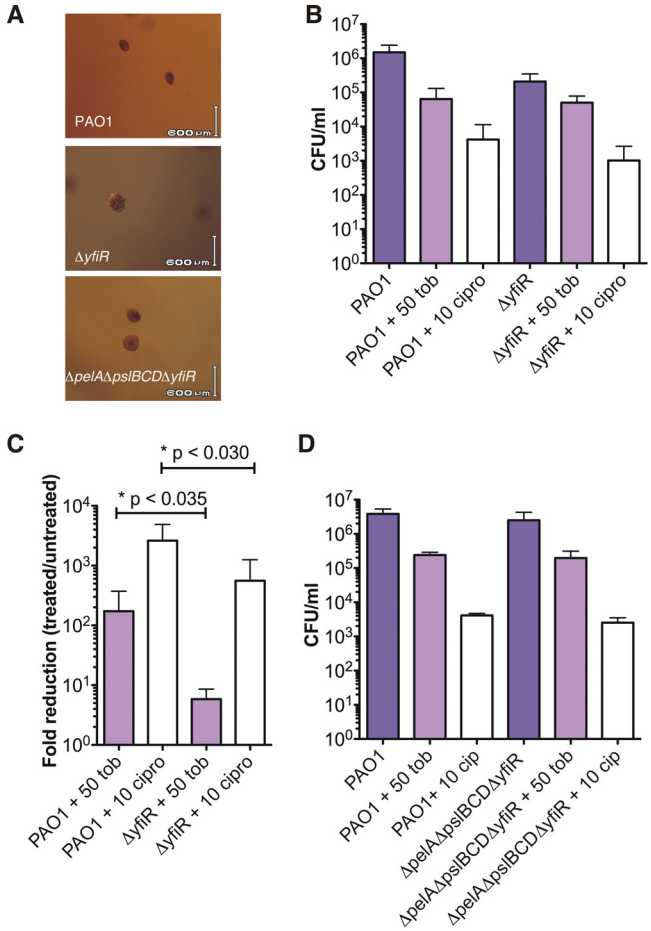

FIG 4 Aggregate morphology and survival of *Pseudomonas aeruginosa* Δ*yfiR* knockout mutant strains overproducing Pel/Psl. (A) Micrographs of PAO1, Δ*yfiR* mutant, and Δ*pelA* Δ*pslBCD* Δ*yfiR* mutant aggregates. (B) Survival of Δ*yfiR* mutant compared to PAO1 after treatment with 50 μg/ml tobramycin (tob) or 10 μg/ml ciprofloxacin (cip or cipro) for 3 h. (C) The difference between the reductions in CFU after antimicrobial treatment is shown for the Δ*yfiR* mutant strain compared to PAO1; $n = 8$. (D) Survival of the Δ*yfiR* Δ*pelA* Δ*pslBCD* mutant strain compared to PAO1 after treatment with 50 μg/ml tobramycin or 10 μg/ml ciprofloxacin for 3 h; $n = 3$. For details, see the Fig. 1 legend.

DISCUSSION

In this study, we show, in agreement with earlier studies (Staudinger at al. [17]), that *P. aeruginosa* strains with or without the ability to produce biofilm matrix components can form biofilm-like aggregates when grown under conditions where motility is restricted. Contrary to previous suggestions, however, we submit that the biofilm matrix plays a role in antimicrobial tolerance of *P. aeruginosa* aggregates. Bacteria in aggregates formed by an alginate-overproducing *mucA* mutant strain showed up to a 50-fold higher tobramycin tolerance than bacteria in aggregates formed by the wild type. Bacteria in aggregates formed by Pel/Psl-overproducing Δ*wspF* and Δ*yfiR* mutant strains exhibited up to 30-fold increased antibiotic tolerance compared to bacteria in wild-type aggregates. Deletion of alginate biosynthesis genes in the *mucA* background and deletion of Pel/Psl biosynthesis genes in the Δ*wspF* and Δ*yfiR* backgrounds reduced the antibiotic tolerance of the aggregates to near-wild-type levels, corroborating that the antibiotic tolerance phenotypes depend on the biofilm matrix components.

We treated *P. aeruginosa* biofilm-like aggregates with two different antimicrobials with different modes of action and physical properties, such as charge. Evidence has been provided that the *P. aeruginosa* biofilm matrix blocks the penetration of charged tobramycin into the biofilm, while this is not the case for uncharged ciprofloxacin (5). Our finding that overproduction of biofilm matrix components increases the tolerance

TABLE 1 Strains used throughout this study

Strain	Description	Source
Pseudomonas aeruginosa		
PAO1	Wild-type sequenced strain	Laboratory collection
Δ*pelA* Δ*pslBCD* mutant	In-frame deletion of *pelA* and *pslBCD*	21
Δ*pelA* Δ*pslBCD* Δ*algD* mutant	In-frame deletion of *pelA*, *pslBCD*, and *algD*	This study
mucA mutant	PAO1 carrying the *mucA22* allele	10
mucA Δ*algD* mutant	*mucA* with in-frame knockout of *algD*	10
Δ*wspF* mutant	In-frame deletion of *wspF*	Joe J. Harrison
Δ*wspF* Δ*pelA* Δ*pslBCD* mutant	In-frame deletion of *wspF*, *pelA*, and *pslBCD*	Joe J. Harrison
Δ*yfiR* mutant	In-frame deletion of *yfiR*	14
Δ*yfiR* Δ*pelA* Δ*pslBCD* mutant	In-frame deletion of *pelA*, *pslBCD*, and *yfiR*	This study
Escherichia coli		
S17		Laboratory collection
DH5a		Laboratory collection

of *P. aeruginosa* aggregates toward both tobramycin and ciprofloxacin may indicate that the presence of the matrix components alters the physiology of the bacteria in the aggregates, e.g., by restricting penetration of nutrients or oxygen into the aggregates.

In addition to increased antimicrobial tolerance, biofilm matrix-overproducing bacteria evidently also display increased immune evasion. Alginate has been shown to protect *P. aeruginosa* against human leukocyte killing (11), in addition to inhibiting pulmonary clearing and increasing bacterial load in a mucine lung infection model (19). Psl expression is also important for reduced phagocytosis (20). In line with this, increased immune evasion of Δ*yfiR* mutant strains has been reported, and it has been shown that disruption of *pel* and *psl* in a Δ*yfiR* mutant strain renders it as sensitive to phagocytosis by macrophages as the wild type (14).

In conclusion, we have provided evidence that overexpression of biofilm matrix components protects *P. aeruginosa* aggregates against antimicrobials. This might explain the strong selection for matrix-overproducing variants in chronic infections that are intensively treated with antibiotics.

MATERIALS AND METHODS

Strains and growth medium. The bacterial strains used in this study, PAO1 and mutant derivatives, are listed in Table 1. All strains were cultivated in LB broth and on 1% LB agar plates.

Strain constructions. The Δ*pelA* Δ*pslBCD* Δ*algD* mutant was constructed by knocking out *algD* from the Δ*pelA* Δ*pslBCD* mutant strain used in a study by Rybtke et al. (21), using the protocol described in reference 22, with primers Up-F (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTCACGGAACTTCCCTCGCAG AG-3′), Up-R (5′-TACCAGCAGATGCCCTCGGCCTGCAAACCAAAGATGCTGATTCGCAT-3′), Down-F (5′-CAG GCCGAGGGCATCTGC-3′), Down-R (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTA-AGTACGGGTAGACCAC GTGG-3′), Seq-F (5′-CATCAAGTTGGTATCAAGTG-3′), and Seq-R: (5′-GGAACACGTGCGACGG-3′).

The Δ*yfiR* deletion construct and the corresponding PAO1 strain were obtained from the lab of Urs Jenal. Deletion of *yfiR* in a Δ*pelA* Δ*pslBCD* background was constructed using the protocol from reference 23 using primers Seq-F (5′-CGGTATAGCTGATGGAACGG-3′) and Seq-R (5′-GAAACCGAAGATCACCAGCTG-3′).

Aggregate cultivation and antibiotic tolerance assessment. *Pseudomonas aeruginosa* aggregates were prepared as described in reference 17 but using LB agar instead of Mueller-Hinton agar. In short, overnight cultures in LB broth of *Pseudomonas aeruginosa* strains were diluted and mixed with 400 μl of molten 0.8% LB agar to allow 100 to 200 cells per gel plug. Gel plugs were cast in the cylinder of single-use 2.5-ml syringes and incubated for 24 h at 37°C. Gel plugs were transferred to 4 ml of saline with or without 50 μg/ml tobramycin or 10 μg/ml ciprofloxacin for 3 h. After treatment, gel plugs were transferred to 600 μl of 0.9% saline in 1.5-ml tubes, 2 marble beads were added, and the gel plugs were disrupted by 2 cycles of 10 s at 6,000 rpm in a MagNA Lyser (Roche). Aggregates were then disrupted by 5 min of degassing and 5 min of sonication in a water bath. Subsequently, the ability of the bacteria to survive antibiotic treatment was assessed by plating on agar plates and counting of CFU after overnight incubation. All mutant strains were run alongside with and compared to the wild-type PAO1 from which they originated.

Statistical analysis. In the figures, error bars indicate the standard deviation (SD) of *n* biological replicates, where $n > 3$, as indicated in the legends. *P* values were determined by Student's *t* test using GraphPad Prism version 5.0.

ACKNOWLEDGMENTS

We are grateful to Joe J. Harrison (University of Calgary) for providing the Δ*wspF* and Δ*wspF* Δ*pelA* Δ*pslBCD* mutant strains.

This work was supported by the Danish Research Council by grant DFF–1323-00177 to T.T.-N. and King Christian X's Foundation to L.G.

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